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# Simple method for clinical determination of 13 carotenoids in human plasma using an isocratic high-performance liquid chromatographic method

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**Abstract**

We report a reversed-phase high-performance liquid chromatography method which resolves 13 identified carotenoids and nine unknown carotenoids from human plasma. A Nucleosil $C_{18}$ column and a Vydac $C_{18}$ column in series are used with an isocratic solvent system of acetonitrile–methanol containing 50 m*M* acetate ammonium–dichloromethane–water (70:15:10:5, v/v/v/v) as mobile phase at a flow-rate of 2 ml/min. The intra-day (4.5–8.3%) and inter-day (1.3–12.7%) coefficients of variation are suitable for routine clinical determinations. 

*Keywords:* Carotenoids

## 1. Introduction

Numerous epidemiological studies suggest that the dietary intake of carotenoid-rich foods is inversely related to the relative risk of cancers and cardiovascular diseases [1]. Because food composition tables report only values for β-carotene content, most of these studies have focused on this sole pigment and numerous analytical methods have been developed for its quantification in plasma and biological tissues [2–4]. Recently, other carotenoids have been suspected to exhibit positive health effect in humans. Hence, a negative association between consumption of tomato and tomato products and the risk of prostate cancer has been reported suggesting that lycopene might be involved in this protective effect [5]. High amounts of lutein and zeaxanthin accumulate in the macula lutea of retina [6] and the consumption of lutein and zeaxanthin-rich vegetables is suggested to protect against age-related macular degeneration [7]. Moreover, lycopene, lutein, canthaxanthin and β-cryptoxanthin have been shown to quench singlet oxygen and scavenge free-radicals as efficiently as β-carotene [8,9]. Consequently, they might prevent deleterious oxidation processes and then contribute to the prevention of several diseases.

At high doses, carotenoids have also been associated to harmful events. Hence supplementation of

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heavy smokers with 20 mg of β-carotene or more for several years resulted in a higher incidence of lung cancer [10,11]. Similarly, daily consumption of drugs (Phenoro) or skin-tanning pills containing high levels of canthaxanthin resulted in golden crystal deposit in the retina and might be associated with alterations of electroretinograms [12].

For epidemiological and clinical purposes, it now appears necessary to develop new methods to separate and quantify a large number of carotenoids in plasma. Xanthophylls (oxygenated carotenes) and carotene isomers are adequately separated using normal- [13], supercritical- [14] HPLC phases, and reversed-HPLC phase associated with gradient [15] or step-by-step [16] elution conditions. However, these methods require specific apparatus and lead in most cases in varying retention times and regular clean-up [17]. Isocratic elution systems were described for carotenoid analysis but they failed to quantify zeaxantin, cantaxanthin, and *cis*-isomers of lycopene and β-carotene [18,19]. We present here a new reversed-HPLC method that separates and quantifies 13 carotenoids including *cis*-and *trans*-isomers of β-carotene and lycopene, lutein, zeaxanthin and canthaxanthin, in human plasma using a single detector. This method does not require a re-equilibration procedure for analytical columns and leads to constant retention time and low variation coefficients (1–12%) of levels of carotenoids measured in plasma.

## 2. Experimental

### 2.1. Chemicals and reagents

Astaxanthin, zeaxanthin, canthaxanthin, β-cryptoxanthin, echinenone, lycopene, α-carotene, 9-*cis*- and 13-*cis*-β-carotene were generously given by Hoffman–La Roche (Basel, Switzerland). Lutein and *all-trans*-β-carotene were purchased from Sigma. Ammonium acetate (7.5 *M*) was supplied by Sigma (l'Isle d'Abeau Chesnes, France). HPLC-grade acetonitrile, methanol, isopropanol, tetrahydrofuran and hexane were obtained from Carlo Erba (Chaussée du Vexin, France). Dichloromethane, stabilized with amylene (25 mg/ml), was purchased from Mallinckrodt (Deventer, Holland). HPLC water was obtained by a MilliQ Plus water purification system (Millipore).

### 2.2. Preparation of standards

Stock solutions of carotenoid standards (1.8–2.1 m*M*) were stored at −20°C in tetrahydrofuran. These solutions were then diluted in isopropanol and then mobile phase (2.5 μ*M*) (working solution). Using the respective extinction coefficient (E1%) [20], their concentrations were determined spectrophotometrically after dilution in ethanol (xantophylls) or hexane (carotenes). Echinenone was used as internal standard.

### 2.3. Chromatographic system

HPLC apparatus consisted in a Waters system equipped with a pump (Waters 610 fluid unit), a regulator (Waters 600 controller), a cooled autosampler (Waters 717 plus) and a UV–visible photodiode-array detector (Waters 996). MILLENIUM 32 software (version 3.05.01) from Waters was used for instrument control, data acquisition and data processing.

Analyses were performed on a 150×4.6 mm, RP $C_{18}$, 3-μm Nucleosil column (Interchim, Montluçon, France) coupled with a 250×4.6 mm RP $C_{18}$, 5-μm, Vydac TP54 (Hesperia, CA) and a 20×4.6 mm $C_{18}$, 5-μm, Hypersil guard column.

The mobile phase consisted in acetonitrile/methanol containing 50 m*M* ammonium acetate/water/dichloromethane (70/15/5/10; v/v/v/v).The methanol fraction was first prepared by dilution of acetate ammonium (7.5 *M*) in methanol. Then this mixture was added to the other of the solvents of the mobile phase.

The flow-rate was 2 ml/min. The run time was set at 50 min. Wavelength detection was set up at 450 nm for carotenoids, 325 nm for vitamin A and 292 nm for vitamin E. Absorption spectra of each standard was measured between 300 and 600 nm in the mobile phase and stored in a spectra library in order to support the identification of plasma carotenoids

### 2.4. Sample extraction

Two human pools (250 ml) were obtained from the local blood transfusion centre (Centre de Transfusion de Clermont-Ferrand), aliquoted in 0.6-ml fractions and stored at −80°C until analysis. All extractions were performed at room temperature under red or yellow light, to minimise light-induced isomerisation. For routine experiments, the following extraction procedure was used. One volume of plasma was deproteinized by addition of one volume of ethanol containing the internal standard (echinenone). Carotenoids were extracted twice by the addition of two volumes of hexane. The mixture was mixed, 30 s, and then centrifuged for 5 min at 500 g. Both upper hexane phases were collected and then evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of dichloromethane/methanol mixture (65/35; V/V), and 80 μl was injected for HPLC analysis.

### 2.5. Quantification

Six-point external standard curves (ranging from 10 to 200 ng) were constructed from diluting working solution of standards in the mobile phase. Plasma concentrations of carotenoids were then calculated using a linear regression $ax + b$ (concentration vs. area) of the six-point external standard curve and were adjusted by percent recovery of the added echinenone internal standard. The precision of the method was assessed through the variation coefficient of intra-day and inter-day repeatability and inter-laboratory calibration procedure which involved five different laboratories.

## 3. Results and discussion

The aim of this study was to separate, by using an isocratic elution system, the main human plasma carotenoids, i.e. lutein, zeaxanthin, β-cryptoxanthin, *all-trans* lycopene, *cis*-lycopene isomers, α-carotene, *all-trans* β-carotene and *cis*-β-carotene isomers, plus canthaxanthin, a carotenoid which can accumulate after oral supplementation or specific dietary habits.

Using complex analytical techniques, Khachik et al. identified 34 different carotenoid structures in serum [21]. However this method is technically difficult and in clinical surveys, reversed-phase HPLC is usually considered more reproducible and simpler to apply for routine determination. Using isocratic elution systems, zeaxanthin, cantaxanthin and *cis*-isomers of lycopene and β-carotene were not quantified [18,19]. We tested carotenoid standards on our nucleosil column alone and zeaxanthin co-eluted with lutein, *cis*-isomers of lycopene co-eluted with *trans*-lycopene, and 9-*cis*-β-carotene co-eluted with *trans*-β-carotene ($\alpha < 1.1$). When we used the Vydac column alone, cantaxanthin co-eluted with zeaxanthin, *cis*-isomers of lycopene and β-carotene co-eluted with their *trans*-isomers ($\alpha < 1.1$). By connecting the two columns in series, we increased the selectivity and zeaxanthin was perfectly separated from lutein ($\alpha = 1.17$) and canthaxanthin ($\alpha = 1.34$), *cis*-isomers were clearly separated from *trans*-lycopene ($\alpha = 1.05$ and $\alpha = 1.18$), and 13-*cis*-β-carotene was separated from *trans*-β-carotene ($\alpha = 1.18$). Fig. 1 shows chromatograms of pure standards (A) and human plasma carotenoids (B–C). All standards were perfectly separated in a 50-min run (Table 1). In most human plasma ($n = 150$), lutein, zeaxanthin, β-cryptoxanthin, total lycopene, α-carotene, *all-trans*-β-carotene and 13-*cis*-β-carotene were clearly identified with respect to both retention time and absorption spectrum in comparison with authentic standards (Table 1).

This method appears as efficient as normal-phase and supercritical HPLC methods to separate lutein from zeaxanthin and *cis*- from *trans*-carotene isomers [13,14].

In one reference plasma, peak 8 (Fig. 1B) was identified as canthaxanthin with respect to both its retention time and its absorption spectra. It was as concentrated as lutein (0.3 1 μ*M*) (Table 2). In this case, the canthaxanthin peak was perfectly separated from zeaxanthin and β-cryptoxanthin. This plasma was obtained from the local blood transfusion centre. We could not get any information about the history of the blood donor for ethical reasons. Canthaxanthin is present in specific foods such as mushrooms (*Cantharellus cinnabarinus*) [22] and trout flesh but only at very low levels (0.14 mg/100 g) [23]. Consequently it can be suggested that daily consumption of very high quantity of these foods would be necessary to result in high canthaxanthin level in

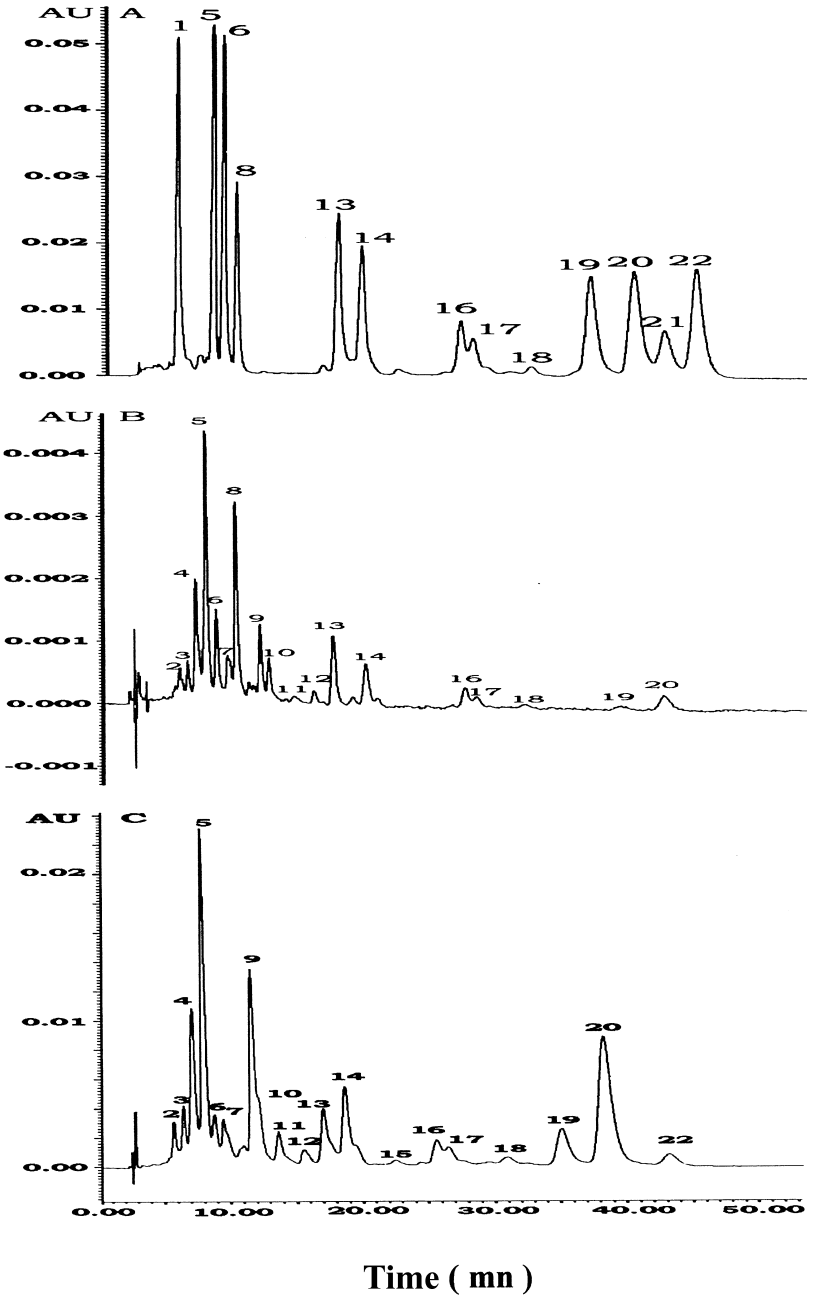


Fig. 1. Chromatograms of pure standards (A) and extracted carotenoids from canthaxanthin rich (B) and control human plasma (C). HPLC conditions: columns, 150×4.6 mm Nucleosil $C_{18}$, 3-μm particle size in series with 250×4.6 mm Vydac TP 254 $C_{18}$, 5-μm particle size, equipped with a 20×4.6 Hypersil column, 5-μm particle size; detection 450 nm; flow-rate 2 ml/min; eluent, acetonitrile–methanol containing 50 m*M* acetate ammonium–dichloromethane–water (70:15:10:5, v/v/v/v). For identification see Table 1.

Table 1
Identification and absorbance maxima in the HPLC solvents of human plasma carotenoids resolved in Fig. 1B

| Peak number | Compound | Retention time (min) | Absorption maxima (nm) | | | |
|---|---|---|---|---|---|---|
| 1 | Astaxanthin | 5.6 | – | – | (476.8)[a] | – |
| 2 | UK1 | 5.8 | 330 | 418.5 | (441.6) | 469.5 |
| 3 | UK2 | 6.5 | 330 | 416.1 | (440.4) | 469.5 |
| 4 | UK3 | 7.2 | 332.1 | – | (445.2) | 473.2 |
| 5 | Lutein | 8.3 | – | – | (446.4) | 474.4 |
| 6 | Zeaxanthin | 9.2 | – | – | (453.7) | 480.5 |
| 7 | UK4 | 9.5 | 330 | 417.3 | (440.4) | 470.7 |
| 8 | Canthaxanthin | 10.2 | – | 420 | (474.4) | |
| 9 | UK5 | 12.2 | – | – | (447.9) | 477 |
| 10 | UK6 | 13.7 | – | – | (452.7) | – |
| 11 | UK7 | 15.6 | – | – | (452.5) | 474.4 |
| 12 | UK8 | 17.1 | – | – | (447.9) | 477 |
| 13 | β-Cryptoxanthin | 18.7 | – | – | (453.7) | 479.3 |
| 14 | Echinenone | 19.8 | – | – | (461) | – |
| 15 | UK9 | 22.5 | – | – | (429.4) | 452.5 |
| 16 | *all-trans* Lycopene | 25.7 | – | 445.2 | (473.2) | 503.6 |
| 17 | *cis*-Lycopene | 26.6 | 360.2 | 445.2 | (473.2) | 503.6 |
| 18 | *cis*-Lycopene | 30.9 | 360.2 | 440.4 | (465.9) | 495.1 |
| 19 | α-Carotene | 37.4 | 338.1 | – | (447.6) | 476.8 |
| 20 | *all-trans* β-Carotene | 40.7 | | – | (454.9) | 480.5 |
| 21 | 9-*cis*-β-Carotene | 43 | 352.5 | – | (448.9) | 476.8 |
| 22 | 13-*cis*-β-Carotene | 45.5 | 339.6 | – | (446.4) | 470.7 |

[a] Values in parentheses represent the higher absorption maximum.

plasma. Canthaxanthin is also included in pharmaceutical drugs (Phenoro, F. Hoffmann–La Roche) used for skin photosensitive disorder treatments, or tanning pills (Orobronze, Applipharm, Marseille, France). It cannot be excluded that the blood donor was under Phenero or Orobronze treatment. Our HPLC method is efficient for plasma canthaxanthin determination, even at high concentration.

No *cis*-lycopene standard is commercially available yet, but absorption spectra analysis, especially in the 340–360 nm range and literature data suggest that at least two *cis*-isomers of lycopene were present in human plasma [24]. These *cis*-isomers eluted just behind the *trans*-lycopene peak. Because *trans*- and *cis*-isomers of lycopene are well resolved in our system, it is possible to quantitate separately each isomer using their respective E1% coefficient [24], and thus to optimise total lycopene determination.

In addition to the 13 identified carotenoids, nine

Table 2
Concentration of carotenoids in one reference human plasma rich in canthaxanthin and variability coefficient of the HPLC analysis

| Carotenoids | mean±SD μ*M* | Equation of the calibration curves | C.V. intra-day ($n=10$) | C.V. inter-day ($n=4$) |
|---|---|---|---|---|
| Lutein | 0.29±0.01 | $20.4x+1.3$ | 4.5 | 4.5 |
| Zeaxanthin | 0.14±0.01 | $20.9x+2.0$ | 5.9 | 1.3 |
| Canthaxanthin | 0.31±0.01 | $24.4x+0.9$ | 7.9 | 4.5 |
| β-Cryptoxanthin | 0.23±0.01 | $18.5x+6.8$ | 7.3 | 2.6 |
| Total lycopene | 0.70±0.08 | $27.9x+9.3$ | 7.0 | 10.9 |
| α-Carotene | 0.05±0.01 | $28.3x+0.6$ | 8.3 | 12.7 |
| β-Carotene | 0.16±0.02 | $24.4x+2.9$ | 7.1 | 9.9 |
| 13-*cis*-β-Carotene | Not detected | $33.6x+0.3$ | – | – |

unknown compounds were separated in the same run. Identification of these compounds is being carried out and mass spectrum analysis will be used for the determination of their chemical structure. The peak area of the two compounds which eluted at 7.2 (UK3) and 12.2 min (UK5) (Fig. 1C, Table 2) were only two-fold lower than that of lutein, and were as high as that of β-cryptoxanthin and lycopene, which are usually considered as the major plasma carotenoids. The maximum of absorption of UK3 were 445 nm, which is close to the absorption maxima of numerous xanthophylls including lutein, and 332 nm which is specific of *cis*-isomers (Fig. 2). In our experimental conditions, the absorption spectra of UK3 is very close to that reported by Khachik et al. [25] for the *cis*-lutein isomer. However, in reversed-phase conditions, *cis*-isomers of lutein usually elute after *trans*-lutein while in the present study UK3 eluted before. Further investigation is thus required to identify this carotenoid. UK5 exhibited an absorption maxima at 448 nm and did not absorb in the UV range. The other UK peaks had a smaller area and absorbed between 429 and 453 nm.

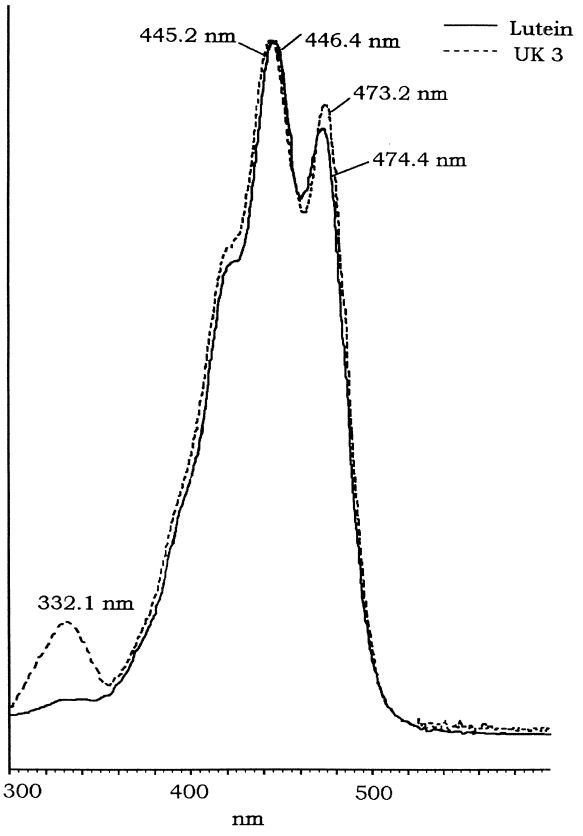


Fig. 2. Absorption spectra of the unknown carotenoid UK3 (----) and pure standard of lutein (—). The spectra were captured on-line through the use of a photodiode array detector. HPLC conditions (see Fig. 1).

In this study, astaxanthin was not detected in any human plasma. By using the same extraction and chromatographic procedures, we were able to separate α-tocopherol from tocopherol acetate, and retinol from retinyl laurate when the detection was set at 292 and 325 nm respectively. Thus by using several detectors in series or a diode array detector, this method can be applied to determine the concentration of vitamins A and E, and 13 carotenoids in human plasma in a single run.

Standard calibration curves were linear up to the maximal quantity we injected (400 ng) and the detection limit was as low as 5 ng for all major carotenoids. Our reference plasma was obtained from the transfusion centre, was aliquoted in 0.6 ml fractions and stored at −80°C until analysis. It was diluted with a phosphate saline buffer in order to estimate the limit of quantification from plasma. The quantification limit was 0.02 μ*M* for lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopene, α-carotene and *trans*-β-carotene. The intra-day repeatability ($n=10$) and the inter-day reproducibility ($n=4$) were measured from analyses of laboratory reference plasma (Table 2). The intra-day and the between-day precision (C.V.), 4.5–8.3% and 1.3–12.7% respectively, were in the acceptable range for clinical analyses.

We have tested the validation parameter of our method through inter-laboratory calibration procedures. Five laboratories analysed the carotenoids from a same reference human plasma and by using its own HPLC methods. Hence, the lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene levels we determined deviated in the range of 1–13% from the mean of the carotenoid concentrations.

In conclusion, we described here a simple isocratic method for the HPLC determination of at least 13 carotenoids in human plasma. When wavelength multidetection can be applied, vitamin A and vitamin E levels can be measured from the same run. We

showed that canthaxanthin, even at high concentration, can be quantified, nine unknown carotenoids or their metabolites are separated but their identification requires further investigation.

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